REMARKS

Claims

Claims 1–5, 17, 21, 22, and 33 are currently pending with claims 6–14, 19, 20, and 23–30 withdrawn from consideration due to election/restriction. Claims 15–16, 18, and 31–32 are cancelled without prejudice or disclaimer. Claim 34 is added by the paper.

Claim Amendments

The claims have been amended in accordance with conventional US practice. No new matter is added.

Rejection under 35 USC §112, second paragraph

Applicants thank the Examiner for his careful reading of the claims. The rejection, not specifically discussed herein, is moot in view of the amendments. Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §101 (utility)

Claims 1–5, 17, 21, 22, and 33 stand rejected under 35 U.S.C. §101 as allegedly lacking an apparent or disclosed specific and substantial credible utility. Applicants respectfully traverse this rejection.

The Office Action at page 4 concedes that Dr. Gottwald's declaration under 37 CFR §1.132 (filed: 01/31/2002) "shows that the complete abolition of a protein of the instant invention from a male mammal results in a reduction in the fertility of that mammal." However, the Office Action proceeds to allege that "this does not appear to support a conclusion that the administration of a protein of the instant invention, or antibodies thereto, to a mammal will affect the fertility of the mammal." The PTO's contention is built around the assumption that "one must know at least *one agonist* to the receptor protein and at least one physiological parameter which is influenced by the *binding of the agonist* to the receptor." This contention is respectfully traversed.

At the outset, the physiological role of the claimed ESRP in reproductive physiology is clear from Applicants' own specification and the disclosure contained in Dr. Gottwald's declaration. With respect to the PTO's contention that disclosure of at least one ligand being crucial to understanding the disclosed role of claimed polypeptides, Applicants submit that this contention is totally unfounded, and moreover not *in line* with current scientific knowledge of GPCRs. For example, GPCRs whose activities are not dependent on *extracellular ligands* (i.e., ligand-independent) are widely recognized in the art. A quick search on PUBMED with the search term "ligand-independent GPCR" reveals more than twenty scientific publications drawn to evidence of such receptors and mechanism(s) of their activity. See, Exhibit A. It is known, for

example, that GPCRs can function in a ligand-independent manner via constitutive activation and/or homo/hetero dimerization. See, also the enclosed publcation by Leeb-Lundberg et al., which relates to B1 bradykinin (BK) receptor (B1R). As disclosed by Leeb-Lundberg, B1R is a seven-transmembrane domain, G protein-coupled receptor, which exhibits a high level of ligand-independent, constitutive activity. Therefore, the Examiner's quest for a ligand is completely unfounded and scientifically baseless.

Applicants' specification discloses a utility of the ESRP polypeptides of the instant invention in the diagnosis and treatment of male infertility. This is stated in the first paragraph of the SUMMARY OF THE INVENTION section of the instant application. A corroborating declaration by Dr. Gottwald under 37 CFR §1.132 (filed: 01/31/2002), which provided scientific evidence of loss of fertility in ESRP knockout mouse, was also provided. This should be sufficient to meet the statutory requirements for utility. Accordingly, the rejection under §101 should be withdrawn.

Rejection under 35 USC §112, first paragraph (written description)

The rejection of claims 1–5, 17, 21, 22, and 33 under 35 U.S.C. § 112, first paragraph as allegedly lacking a written description is respectfully traversed.

In view of the aforementioned arguments and Applicants' remarks filed February 15, 2007, it is courteously submitted that Applicants' claims in the current form, with adequate support from the specification and the references cited therein, fully comply with the statutory requirements under 35 U.S.C. § 112, first paragraph, as specified in the PTO's own guidelines. Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §102 (b) in view of Osterhoff et al.

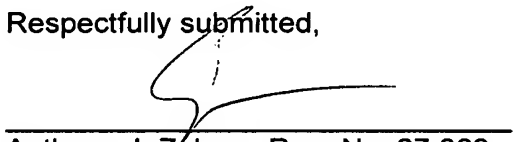
Claims 1–5, 17, 21, 22, and 31–33 stand rejected under 35 U.S.C. § 102(b) as being allegedly anticipated by Osterhoff et al. (DNA and Cell Biol., vol. 16, pages: 379–389, 1997). Applicants respectfully traverse this rejection

Osterhoff et al. is the Applicant's own publication, which was published April 1997, i.e., within a year prior to the filing of the parent application (Serial No.: 09/041,745). Applicants submit that the parent application provides an enabling disclosure of the presently claimed invention and provides an adequate written description of the presently claimed invention. Applicants further submit that the parent, like the instant application, meets the requirements of 35 U.S.C. §112, first paragraph, such that benefit of the parent application should be accorded, along with withdrawal of the rejection under35 U.S.C. §102(b).

In view of the above and attached, it is respectfully submitted that the claims are in condition for allowance. However, should the Examiner have any questions or comments, he is cordially invited to telephone the undersigned at the number below.

The Commissioner is hereby authorized to charge any fees associated with this response to Deposit Account No. 13-3402.

Respectfully submitted,



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Attorney Docket No.: **SCH-2029**

Date: **August 23, 2007**

Encl:
Publication by Leeb-Lundberg et al. (*Journal of Biological Chemistry*, 2001)

EXHIBIT A

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Items 1 - 25 of 25 One page.

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The Human B1 Bradykinin Receptor Exhibits High Ligand-independent, Constitutive Activity

ROLES OF RESIDUES IN THE FOURTH INTRACELLULAR AND THIRD TRANSMEMBRANE DOMAINS*

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The B1 bradykinin (BK) receptor (B1R) is a seven-transmembrane domain, G protein-coupled receptor that is induced by injury and important in inflammation and nociception. Here, we show that the human B1R exhibits a high level of ligand-independent, constitutive activity. Constitutive activity was identified by the increase in basal cellular phosphoinositide hydrolysis as a function of the density of the receptors in transiently transfected HEK293 cells. Several B1R peptide antagonists were neutral antagonists or very weakly efficacious inverse agonists. Constitutive B1R activity was further increased by alanine mutation of Asn^{121} in the third transmembrane domain of the receptor ($B1A^{121}$). This mutant resembled the agonist-preferred receptor state since it also exhibited increased agonist affinity and decreased agonist responsiveness. A dramatic loss of constitutive activity occurred when the fourth intracellular C-terminal domain (IC-IV) of the human B2 BK receptor subtype (B2R), which exhibits minimal constitutive activity, was substituted in either B1R or $B1A^{121}$ to make B1(B2ICIV) and B1(B2ICIV)A^{121}, respectively. Activity was partially recovered by subsequent alanine mutation of a cluster of two serines and two threonines in IC-IV of either B1(B2ICIV) or B1(B2ICIV)A^{121}, a cluster that is important for B2R desensitization. The ligand-independent, constitutive activity of B1R therefore depends on epitopes in both transmembrane and intracellular domains. We propose that the activity is primarily due to the lack of critical epitopes in IC-IV that regulate such activity.

The B1 and B2 bradykinin (BK)[1] receptors are seven-transmembrane domain, G protein-coupled receptors (GPCR) (1, 2), which mediate the actions of kinins (3), pro-inflammatory peptides formed in response to tissue injury from kininogen precursors (4, 5). Kinin actions include pain, inflammation, and hyperalgesia (4, 6). The B2 receptor mediates the actions of BK and Lys-BK or kallidin (KD), the first set of bioactive kinins formed following injury, whereas the B1 receptor is thought to mediate the actions of des-Arg^9-BK and des-Arg^{10}-KD, the carboxypeptidase products of BK and KD and the second set of bioactive kinins formed (3). Despite binding very similar ligands, leading to their classification as receptor subtypes, these receptors exhibit only 36% identity. Furthermore, the B2 receptor is constitutively expressed, whereas the B1 receptor is expressed at very low levels, if at all, in healthy tissue but is induced by inflammatory stimuli such as interleukin-1β (2, 7–9) and by kinins themselves (8, 10). This pattern of expression is consistent with the fact that the B2 receptor appears to be the principal kinin receptor under healthy conditions and in the acute stage of the inflammatory response (4, 6, 11, 12), whereas the B1 receptor is important primarily in the chronic stage of the response (6, 13, 14).

The rationale for restricting B1 receptor expression primarily to conditions of injury is not understood. One explanation is that this receptor serves to provide a sustained kinin signal (7), which may be necessary in inflammation but detrimental under healthy conditions. This explanation is consistent with the fact that the B2 receptor response is transient and rapidly desensitizes upon agonist stimulation, whereas the B1 receptor response is sustained and desensitizes very slowly (15, 16). However, restricting B1 receptor expression to prohibit sustained agonist stimulation seems unnecessary since kinin production in the absence of injury is probably low, and the *in vivo* production of the B1 receptor agonists des-Arg^9-BK and des-Arg^{10}-KD by carboxypeptidases from BK and KD, respectively, is relatively inefficient (17). On the other hand, limiting B1 receptor expression would be important if this receptor is constitutively active since constitutive GPCR activity, which occurs both spontaneously and in response to mutations in these receptors, can lead to disease (18, 19) including tumorigenesis (20, 21).

The localization of many natural and unnatural constitutively activating GPCR mutations in transmembrane domains has led to the belief that interhelical contacts are important in regulating the ligand-independent isomerization of these receptors between the inactive and the activated states and, consequently, constitutive activity (22). Residues at a position about two helical turns into TM-III, occupied by an asparagine in the B2 BK (23) and AT1 angiotensin II receptors (24) and by a cysteine in the α_1-adrenergic receptor (25) and glutamate in rhodopsin (26), are thought to participate in such a contact. It has been reported that epitopes in IC-IV also regulate constitutive GPCR activity (27–31), yet little is known about the underlying mechanism. We recently reported that the B2 receptor exhibits minimal ligand-independent activity. Mutation of a cluster of serines and threonines in IC-IV, which is important for agonist-promoted B2 receptor phosphorylation and internalization (32) and desensitization (31), significantly elevated this activity (31). In the present study, we show that the human B1 receptor exhibits a high level of constitutive activity

* This work was supported by National Institutes of Health Grant GM41659. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BK, bradykinin; GPCR, G protein-coupled receptor; KD, kallidin; PI, phosphoinositide; WT, wild-type; IC-IV, fourth intracellular domain; TM-III, third transmembrane domain; DMEM, Dulbecco's modified Eagle's medium; I_A, index of agonist-promoted receptor activity; I_B, index of basal receptor activity.

in HEK293 cells. B2R IC-IV strongly suppresses B1R activity, and this occurs independently of the activation state of the receptor. The suppression is partially due to the presence of critical serines and threonines, but other epitopes are also involved. Thus, we propose that the high constitutive B1R activity is due to the lack of regulatory epitopes in IC-IV.

EXPERIMENTAL PROCEDURES

Materials—[*prolyl*-3,4-^{3}H]NPC17731 (53.5 Ci/mmol), des-Arg^{10}-[3,4-*prolyl*-3,4-^{3}H]kallidin (69–105 Ci/mmol), des-Arg^{10}-[Leu^9][3,4-*prolyl*-3,4-^{3}H]kallidin (67–105 Ci/mmol), and *myo*-[^{3}H]inositol (10–20 Ci/mmol) were obtained from PerkinElmer Life Sciences. M2 monoclonal antibodies against the FLAG epitope were obtained from Eastman Kodak and Sigma. Des-Arg^{10}-kallidin and des-Arg^{10}-[Leu^9]kallidin were from Bachem (Torrance, CA), whereas HOE140 and des-Arg^{10}-HOE140 were from Peninsula (Belmont, CA). NPC17731 and NPC18565 were gifts from D. J. Kyle, Purdue Pharma, Princeton, NJ. R715 was a gift from D. Regoli, Universite de Sherbrooke, Quebec, Canada. B9858 was a gift from F. Marceau, L'Hotel-Dieu de Quebec, Quebec, Canada. The original human and rabbit B1 receptor and human B2 receptor cDNAs were from F. Hess, Merck Research Laboratories, West Point, PA. All other chemicals were obtained as described previously (33, 34).

Mutation and Transfection—Mutations were done using a polymerase chain reaction-ligation-polymerase chain reaction protocol as described previously (33). The FLAG epitope was inserted at the receptor N terminus immediately following the initial methionine. HEK293 human embryonic kidney cells were grown in DMEM supplemented with 10% heat-inactivated horse serum in 10% CO_2 at 37 °C. The cells were transiently transfected with varying amounts of DNA using the calcium phosphate precipitate method as described previously (32).

Particulate Preparation—Transfected HEK293 cells were washed twice with ice-cold phosphate-buffered saline and then pelleted by centrifugation at 2000 × *g* for 10 min. The cells were then resuspended in a buffer containing 25 mM 2-{[2(hydroxymethyl)ethyl]amino}ethanesulfonic acid, pH 6.8, 0.5 mM EDTA, 0.2 mM $MgCl_2$, and 1 mM 1,10-phenanthroline and homogenized using an T25 Ultra-Turrax tissue homogenizer (IKA-Works, Wilmington, NC) at 20,500 rpm for 10 s. Membranes were isolated by centrifugation at 45,000 × *g* for 30 min at 4 °C and washed 1–3 times in the above buffer depending on the experiment. The pellets were then resuspended in the same buffer supplemented with 0.1% bovine serum albumin and 0.014% bacitracin (binding buffer) and used immediately. The preparation was washed one time in the experiments determining the pharmacological profiles of agonist and antagonist binding to the B1 and B2 receptors (Figs. 1 and 4*A* and Table I). In all other binding experiments, the preparation was washed three times. Increasing the number of washes from one to three resulted in an ~10-fold increase in the value of the binding constants, K_d and K_i, for des-Arg^{10}-KD on the various B1 receptor constructs.

Radioligand Binding—Radioligand binding assays were performed essentially as described previously (31, 33). Receptor density was determined on intact HEK293 cells by incubating cells in Leibovitz's L-15 medium, pH 7.4, 0.1% bovine serum albumin including the protease inhibitors bacitracin (140 μg/ml) and 1,10-phenanthroline (1 mM) and a saturating concentration of [^{3}H]des-Arg^{10}-KD or [^{3}H]NPC17731 (3–5 nM) at 4 °C for 60–90 min. Competition binding was done on particulate preparations by incubating the preparations in binding buffer including ~0.2–0.5 nM [^{3}H]des-Arg^{10}-KD or [^{3}H]des-Arg^{10}-[Leu^9]KD with and without various concentrations of competitor at 25 °C for 60–90 min.

Receptor Activity—Activities of various receptor constructs were assayed by monitoring PI hydrolysis in HEK293 cells (31). PI hydrolysis was assayed in cells pre-labeled with 1 μCi/ml *myo*-[^{3}H]inositol in DMEM containing 5% heat-inactivated horse serum. After washing, the cells were incubated in Leibovitz's L-15 medium containing 5 mM LiCl in the absence and presence of an agonist or antagonist for 30 min at 37 °C. When agonists and antagonists were coincubated, the antagonist was added 5 min before the addition of the agonist and then further incubated for 30 min.

Immunoprecipitation and Immunoblotting—HEK293 cells transfected with FLAG epitope-tagged receptors were subjected to immunoprecipitation and immunoblotting essentially as previously described (34). In short, cells were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM sodium phosphate, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) for 30 min at 4 °C. The lysate was centrifuged at 13,000 × *g* for 15 min at 4 °C. The supernatant (1 ml) was then incubated 12–18 h with anti-FLAG M2 antibody (1:200) followed by incubation with protein A-Sepharose beads precoupled to rabbit anti-mouse IgG for an additional 2 h at 4 °C. The beads were then washed with 2 × 1 ml of lysis buffer and then with 1 ml of 10 mM Tris-HCl, pH 7.4. The pellet was heated in SDS-polyacrylamide gel electrophoresis buffer containing 6% β-mercaptoethanol for 5 min at 100 °C and then electrophoresed on 12% polyacrylamide gels. The gel was then electroblotted onto 0.45-μm nitrocellulose membranes and stained with anti-FLAG M2 antibody (1:1000). Immunoreactive bands were visualized with an immunodetection kit using peroxidase-labeled sheep anti-mouse antibody according to the procedure described by the supplier (PerkinElmer Life Sciences).

Data Analysis—Where indicated, data are presented as the mean ± S.E. and were compared using the Student's *t* test. The receptor binding constants, K_d and K_i, for various ligands were calculated by the Radlig program (Biosoft, Ferguson, MO) using data from radioligand binding experiments.

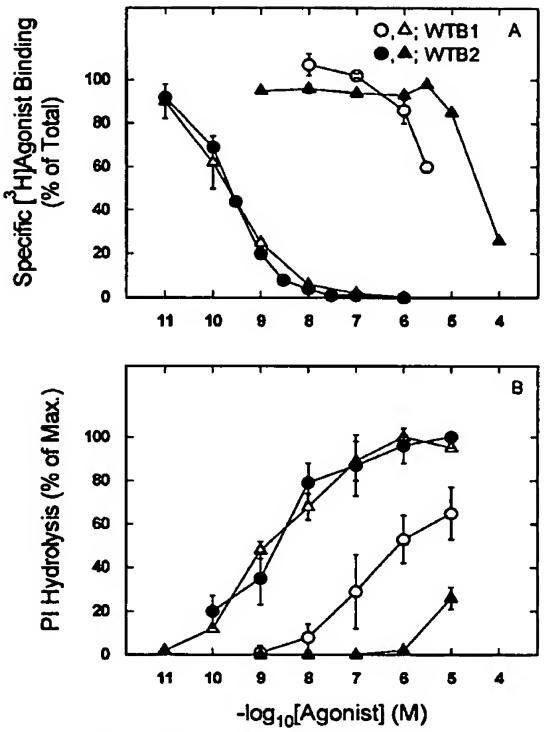

FIG. 1. **Pharmacological profiles of the human B1 and B2 receptors.** *A*, particulate preparations of HEK293 cells transfected with WT B1 (*open symbols*) or WT B2 (*closed symbols*) receptors were washed one time, incubated in the absence and presence of increasing concentrations of des-Arg^{10}-KD (▲, △) and BK (●, ○) as indicated, and assayed for radioligand binding with K_d concentrations of [^{3}H]des-Arg^{10}-KD (WT B1) or [^{3}H]BK (WT B2). *B*, intact cells transfected with WT B1 or WT B2 receptors were incubated with increasing concentrations of des-Arg^{10}-KD and BK as indicated in *A* and assayed for PI hydrolysis. *B*, the results are presented as percentage of maximum, where 100% maximum is the maximum response of the B1 and B2 receptors to des-Arg^{10}-KD and BK, respectively. The results are averages ± S.E. of three independent experiments with each point assayed in duplicate.

RESULTS

Agonist Profile of the Human B1 Receptor Expressed in HEK293 Cells—The human WT B1 receptor transiently expressed in HEK293 cells exhibited a typical agonist profile as determined by both receptor binding and receptor-mediated PI hydrolysis. Fig. 1, *A* and *B*, shows that des-Arg^{10}-KD, a B1 receptor-selective agonist, bound to the B1 receptor with an affinity (K_d = 0.098 nM) and stimulated PI hydrolysis with a potency (EC_{50} = 1.2 nM) which were ~4 and 3 orders of magnitude, respectively, higher than those of BK (K_d = 2880 nM,

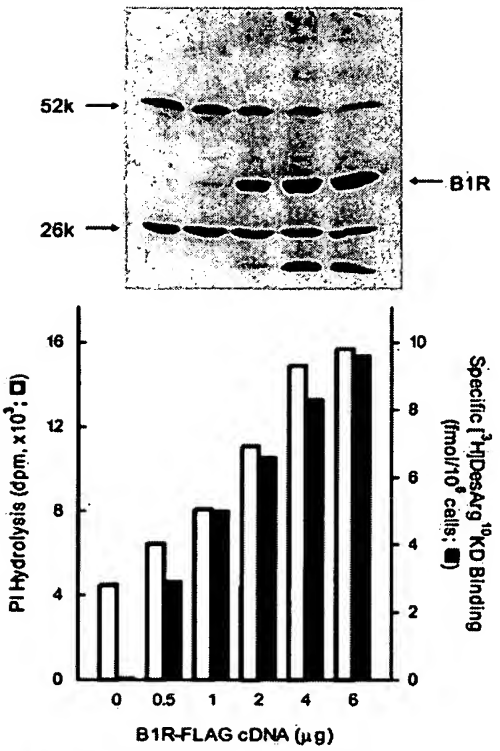

FIG. 2. **Ligand-independent PI hydrolysis by the human B1 receptor.** HEK293 cells were transfected with increasing amounts of B1R-FLAG DNA as indicated. Parallel sets of cells were then assayed for basal cellular PI hydrolysis (*open bars*), specific [^{3}H]des-Arg^{10}-KD binding using a saturating concentration of radioligand (*filled bars*), and anti-B1R-FLAG immunoreactivity (*top*). The position of the receptor in the Western blot is indicated (*right side arrow*). The molecular weights of the IgG heavy and light chains are indicated (*left side arrows*). The results are from a representative experiment performed three times.

EC_{50} = 630 nM), a B2 receptor-selective agonist. This profile was clearly different from that of the human WT B2 receptor subtype transiently expressed in HEK293 cells to which BK bound with an affinity (K_d = 0.190 nM) and stimulated PI hydrolysis with a potency (EC_{50} = 2.0 nM) which were >4 orders of magnitude higher than those of des-Arg^{10}-KD (K_d >5000 nM, EC_{50} >5000 nM).

Ligand-independent and Agonist-dependent Activities of the Human B1 Receptor—Ligand-independent, constitutive B1 receptor activity was monitored by assaying for basal cellular PI hydrolysis as a function of the density of the receptors in transiently transfected HEK293 cells. Fig. 2 shows a representative experiment in which cells were transfected with increasing amounts of cDNA (0.5–6 μg) of a FLAG-tagged human B1 receptor (B1R-FLAG) and then assayed in parallel for receptor expression by specific [^{3}H]des-Arg^{10}-KD binding and anti-FLAG immunoblotting and for ligand-independent activity by basal PI hydrolysis. PI hydrolysis increased with increasing amounts of des-Arg^{10}-KD binding and the B1 receptor-specific 35-kDa peptide. Fig. 3*A* shows that the slope factor of the increase in PI hydrolysis as a function of the human WT B1 receptor, which may be considered as an index of ligand-independent receptor activity and which we previously termed the index of basal activity, or I_B (31), was 0.58. By comparison, the human B1 receptor activity was considerably higher than that of the human B2 receptor, which had an I_B of 0.03 (31). These results show that the B1 receptor is constitutively active, and this activity is evident at receptor densities that would be expected to occur *in vivo*. Similar results were obtained with the rabbit WT B1 receptor indicating that the constitutive activity was not a peculiarity of the human receptor (data not shown).

Addition of the agonist des-Arg^{10}-KD at 1 μM, a concentration sufficient to activate maximally the human B1 receptor, to B1 receptor-expressing cells yielded a slope factor of PI hydrolysis of 16.0. This factor, which we previously termed the index of agonist-promoted receptor activity, or I_A (31), represented a 27-fold increase over the I_B for the B1 receptor (Fig. 3*B*). By comparison, a similar increase (23-fold) was observed following addition of 1 μM BK to the B2 receptor-expressing cells (I_A = 0.86) (31).

The Effect of Antagonists on Ligand-independent and Agonist-dependent Activities of the Human B1 Receptor—GPCR antagonists range in their actions from partial agonism to neutral antagonism to inverse agonism. Inverse agonism requires ligand-independent activity and is thought to involve the stabilization of the inactive receptor state. Several B1 receptor peptide antagonists have been developed (Table I). These peptides bound to the human B1 receptor expressed in HEK293 cells with an order of potency that was NPC18565 > B9858 ~ des-Arg^{10}[Leu^9]KD ~ R715 ~ des-Arg^{10}HOE140 > des-Arg^9[Leu^8]BK (Fig. 4*A* and Table I). As expected, all the peptides, at 1 μM (except des-Arg^9[Leu^8]BK which was at 10 μM), antagonized B1 receptor-mediated stimulation of PI hydrolysis in response to 0.1 μM des-Arg^9-BK in these cells (Fig. 4*B*, *left panel*). On the other hand, none of the peptides significantly inhibited the constitutive B1 receptor activity, even though des-Arg^{10}-[Leu^9]KD, des-Arg^9[Leu^8]BK, and NPC18565 appeared to have some effect (Fig. 4*B*, *right panel*). Thus, these peptides are neutral antagonists or, at most, very weakly efficacious inverse agonists on the human B1 receptor in HEK293 cells.

The Role of Asn^{121} in TM-III in Ligand-independent and Agonist-dependent Human B1 Receptor Activity—Fig. 5*A* shows the position of Asn^{121} in TM-III of the human B1 receptor. This residue is conserved in some GPCR, and mutation of residues at this position in several GPCR results in the constitutive activation of the receptors (22–26). These observations have led to the belief that these residues participate in an interhelical contact with residues in other transmembrane domains that restrains the receptor in an inactive conformational state. One possibility is that the alignment of this residue is different in the B1 receptor resulting in a weaker contact, *i.e.* mutation of Asn^{121} does not lead to an increase in constitutive activity. Fig. 5*B* shows that B1A^{121} had a slope factor of PI hydrolysis that was 9-fold higher than that of the WT B1 receptor. Thus, despite the constitutive activity of the WT B1 receptor, Asn^{121} remains involved in conformational selection in this receptor.

To determine whether the preferred conformation of B1A^{121} is related to that normally favored by the agonist des-Arg^{10}-KD, we studied various parameters of des-Arg^{10}-KD interaction with the receptor. Des-Arg^{10}-KD stimulation elevated the slope factor of PI hydrolysis for this mutant only 3-fold compared with 27-fold for the WT receptor (Fig. 5*B*). Furthermore, the K_d value of des-Arg^{10}-KD binding and the K_i value of des-Arg^{10}-KD to compete with the binding of the antagonist des-Arg^{10}-[Leu^9]KD to this construct were 3.3- and 5.5-fold, respectively, lower than for the WT receptor (Fig. 5, *C* and *D* and Table II). In contrast, the K_d of the antagonist des-Arg^{10}-[Leu^9]KD was not significantly perturbed by this mutation (Fig. 5*C*).

GTP sensitivity of agonist binding signifies the ability of GPCR to isomerize between conformational states of different

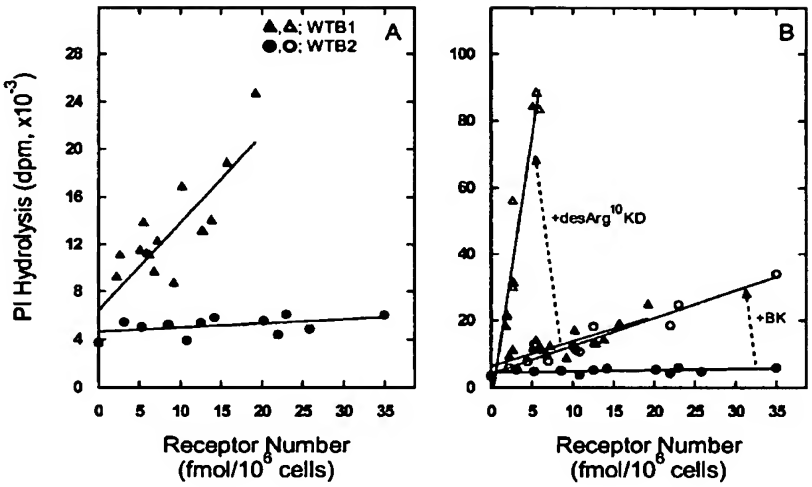

FIG. 3. **Ligand-independent and agonist-dependent PI hydrolysis by the human B1 and B2 receptors.** HEK293 cells transfected with varying amounts of the WT B1 (▲, △) or WT B2 (●, ○) receptors were incubated in the absence (*A* and *B*, *closed symbols*) and presence (*B*, *open symbols*) of 1 μM des-Arg^{10}-KD and 1 μM BK, respectively, and then assayed for PI hydrolysis and radioligand binding using a saturating concentration of radioligand. *B*, the effects of des-Arg^{10}-KD and BK on the B1 and B2 receptor, respectively, are shown (*dashed arrows*). Note the difference in the *y* axis scale in *A* and *B*. The results are from 4 to 8 independent experiments with each point performed in duplicate.

TABLE I
Amino acid sequences of B1 receptor peptide antagonists and their binding constants on WT B1 receptors

Peptide	Sequence[a]	K_i[b]
		nM
Des-Arg^{10}[Leu^9]KD	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	0.998 ± 0.298
Des-Arg^9[Leu^8]BK	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	51.8 ± 1.3
NPC18565	D-Arg-Arg-Pro-Hyp-Gly-Phe-Ser-*X*-Oic	0.071 ± 0.009
Des-Arg^{10}-HOE140	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic	1.25 ± 0.22
R715	Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-β-D-Nal-Ile	0.814 ± 0.199
B9858	Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Tic-Ile	0.672 ± 0.371

[a] Hyp, 4-hydroxy-L-proline; *X*, D-Hype(*trans*-propyl); Oic, octahydroindol-2-carboxylic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Thi, β-2-thienylalanine; Ac, *N*-acetyl; β-Nal, β-2-naphthylalanine; Igl, α-(2-indanyl)glycine.

[b] Particulate preparations of transiently transfected cells were washed one time prior to assay. K_i values were determined by the Radlig program in competition binding experiments (Fig. 4*A*) using a constant concentration of the radiolabeled agonist [^{3}H]des-Arg^{10}-KD and increasing concentrations of the various peptide antagonists. The values are presented as the average ± S.E. of at least three experiments.

degrees of activity and G protein coupling. Inclusion of 100 μM Gpp(NH)p caused an increase (2.2-fold) in the K_i value of des-Arg^{10}-KD to compete with the binding of des-Arg^{10}-[Leu^9]KD binding to the WT B1 receptor (Fig. 5*D* and Table II). This classical result indicates that the agonist prefers to bind to and favor the formation of the G protein-coupled state of the WT B1 receptor and that it is capable of isomerizing between G protein-coupled and -uncoupled states. Gpp(NH)p had no effect on the K_i value of des-Arg^{10}-[Leu^9]KD to compete with the binding of the des-Arg^{10}-KD to the WT receptor (Fig. 5 and Table II), an observation compatible with the behavior of des-Arg^{10}-[Leu^9]KD as a neutral antagonist, *i.e.* it is unable to distinguish between G protein-coupled and -uncoupled receptor states. In terms of B1A^{121}, inclusion of 100 μM Gpp(NH)p caused an increase (2.1-fold) in the K_i value of des-Arg^{10}-KD to compete with the binding of des-Arg^{10}-[Leu^9]KD binding (Fig. 5*D* and Table II). The increase was not significant indicating that it is still unclear whether or not this mutant is readily capable of isomerizing between conformational states.

Taken together, the above results imply that mutation of Asn^{121} in the B1 receptor favors an activated conformation of the receptor. The decrease in des-Arg^{10}-KD responsiveness of and increase in des-Arg^{10}-KD affinity for B1A^{121} strongly suggest that the favored conformation is the one preferred by des-Arg^{10}-KD. Given that the B1 receptor was sensitive to mutation of Asn^{121}, this residue is most likely aligned properly in this receptor. Consequently, other reasons may be responsible for the elevated ligand-independent activity of this receptor.

The Role of IC-IV in Ligand-independent and Agonist-dependent Human B1 Receptor Activity—The human B2 receptor contains a cluster of serines and threonines (Ser/Thr cluster), including Thr^{342}, Thr^{345}, Ser^{346}, and Ser^{348} in IC-IV, that is conserved among B2 receptors from four different species (Fig. 6), and this cluster is important for regulating agonist-promoted receptor activity by receptor phosphorylation and internalization (32) and desensitization (31). Alanine mutation of this cluster significantly increased ligand-independent B2 receptor activity leading to the thought that it is also important for regulating constitutive receptor activity (31). The human B1 receptor contains several serines and threonines in IC-IV including Thr^{321}, Thr^{331}, Ser^{339}, Ser^{340}, and Ser^{341} that are conserved in the rabbit receptor (Fig. 6). On the other hand, the rat and mouse B1 receptors, being severely truncated in their IC-IV, only contain a threonine corresponding to Thr^{321}. Furthermore, the human B1 receptor does not appear to serve as a substrate for phosphorylation (35). Thus, another reason for the high constitutive activity of the B1 receptor could be that this receptor lacks critical serines and threonines in IC-IV that regulate such activity. To test this possibility, we first substituted IC-IV of the B2 receptor, which contains the Ser/Thr cluster, in the B1 receptor to make B1(B2ICIV). As shown in Fig. 7*A*, this substitution dramatically suppressed the constitutive B1 receptor activity. The agonist-dependent activity was also suppressed (Fig. 7*B*). This substitution also inhibited the enhanced constitutive activity and agonist-dependent activity of B1A^{121} (Fig. 7, *C* and *D*). Subsequent alanine mutation of the Ser/Thr clusters in both B1(B2ICIV), to make B1(B2ICIVA$^{Ser/Thr}$), and B1(B2ICIV)A^{121}, to make B1(B2ICIVA$^{Ser/Thr}$)A^{121}, significantly restored the receptor activities, including both the ligand-independent (Fig. 7, *A* and *C*) and agonist-dependent activities (Fig. 7, *B* and *D*). Thus, the Ser/Thr cluster in B2 IC-IV represents one epitope that negatively regulates constitutive receptor activity. Furthermore, this regulation appears to occur independently of the activation state of the receptor

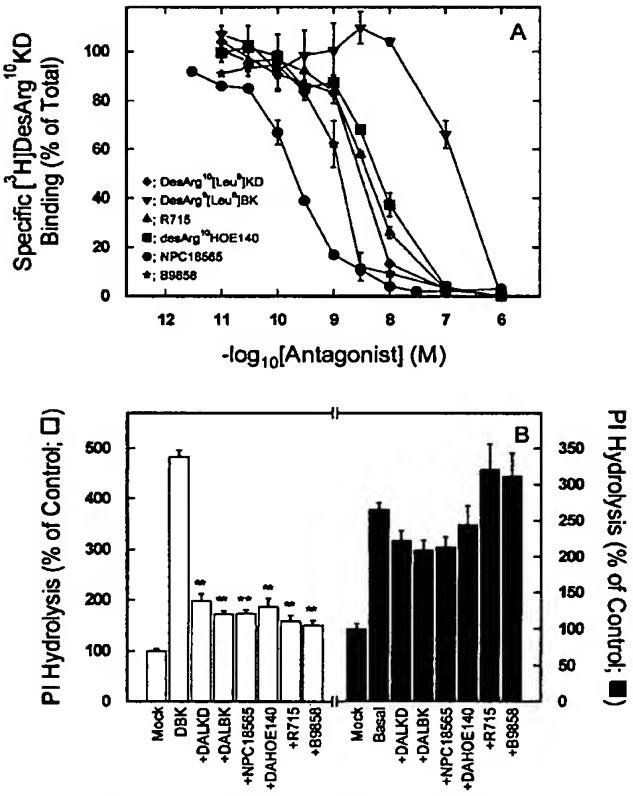

FIG. 4. **Effects of antagonists on ligand-independent and agonist-dependent PI hydrolysis of the human B1 receptor.** *A*, particulate preparations of HEK293 cells transfected with WT B1 receptors were washed one time, incubated in the absence and presence of increasing concentrations of various peptide antagonists as indicated, and assayed for competition radioligand binding using a K_d concentration of [^{3}H]des-Arg^{10}-KD. The results are averages ± S.E. of three independent experiments with each point assayed in duplicate. Binding constants (K_i) calculated from these experiments are summarized in Table I. *B*, HEK293 cells were transfected with WT B1 receptors and incubated with and without 1 μM des-Arg^{10}-[Leu^9]KD (DALKD), 10 μM des-Arg^9-[Leu^8]BK (DALBK), 1 μM NPC18565, 1 μM des-Arg^{10}-HOE140 (DAHOE140), 1 μM R715, and 1 μM B9858 in the presence (*left panel*) and absence (*right panel*) of 0.1 μM des-Arg^9-BK (*DBK*) as indicated and described under "Experimental Procedures." The cells were then assayed for PI hydrolysis. The results are presented as percentage of control where 100% control is the PI hydrolysis in mock-transfected cells and was 5264 ± 390 dpm (n = 11). The density of the receptors was ~15 fmol/10^6 cells. The results are averages ± S.E. of at least six independent determinations with each point assayed in duplicate. **, $p < 0.01$ compared with des-Arg^9-BK (*left panel*).

since it was observed both with and without the Asn^{121} → Ala activating mutation.

DISCUSSION

In this report, we show that the human B1 BK receptor subtype exhibits high ligand-independent, constitutive activity. Despite the constitutive activity of the WT receptor, alanine mutation of Asn^{121} in TM-III results in a further increase in receptor activity. On the other hand, substitution with IC-IV of the B2 receptor, which exhibits minimal ligand-independent activity, strongly suppresses constitutive activity of the B1 receptor as well as the mutation-enhanced activity of B1A^{121}. The suppressive effect of B2 IC-IV is partially due to the presence of serines and threonines that are important for B2 receptor desensitization, but other epitopes also seem to be involved. These observations show that constitutive activity depends on epitopes in both transmembrane and intracellular domains. We propose that the high constitutive B1 receptor activity is due primarily to the lack of epitopes in IC-IV that regulate such activity.

Constitutive receptor activity provides a rationale for restricting the expression of the B1 receptor primarily to conditions of inflammation. Indeed, the I_B for the B1 receptor is almost as high as the I_A for the B2 receptor. It is unlikely that this activity completely substitutes for agonist stimulation of the receptor. In fact, the ability to promote the formation of a receptor state with features of a des-Arg^{10}-KD-preferred state by the Asn^{121} → Ala mutation argues that this agonist has a role in B1 receptor function. However, since the formation of B1 agonists is relatively inefficient (17), constitutive receptor activity may serve a role prior to the formation of a sufficient amount of agonist or, similarly, in anatomical areas where receptors are required but insufficient amounts of agonists are formed. In addition to sustaining the receptor signal, constitutive receptor activity may synergize with interleukin-1β to induce rapidly the receptor, as described previously for receptor agonists (8).

The models explaining ligand-independent, constitutive GPCR activity assert that these receptors spontaneously isomerize between inactive and activated conformational states termed R and R*, respectively, and that R* associates with a G protein to form R*G that triggers the intracellular signal (36, 37). In these models, agonists act by preferentially binding to and stabilizing R*. Even though the basic features of these models prevail, there is emerging evidence that the above equilibrium is not limited to two receptor states but harbors multiple microscopic equilibria with a number of partially/fully activated states. The isomerization constant J that governs the spontaneous formation of the activated state in these models is an important determinant of the level of constitutive activity of a GPCR. The transmembrane location of several natural and unnatural constitutively activating mutations suggests that this process involves the breaking of interhelical contacts necessary to constrain the receptor in an inactive state (22). Residues at a position in TM-III occupied by an asparagine in the AT$_1$-angiotensin II receptor (Asn^{111}) and the B2 BK receptor (Asn^{113}) have been proposed to participate in such a contact since their mutation yields an increase in ligand-independent activity (23, 24). This residue is conserved in the B1 receptor (Asn^{121}). Alanine mutation of this residue led to a 9-fold increase in the ligand-independent receptor activity on PI hydrolysis. This mutation also caused the fold stimulation of receptor activity by the agonist des-Arg^{10}-KD to decrease from 27- to 3-fold, the K_d value of des-Arg^{10}-KD to decrease 3.3-fold, and the K_i value of des-Arg^{10}-KD for competing with the binding of the antagonist des-Arg^{10}-[Leu^9]KD to decrease 5.5-fold. The lack of an effect of the mutation on the K_d value of the antagonist des-Arg^{10}-[Leu^9]KD indicates that this is an agonist-specific effect. The resemblance of this mutant to a des-Arg^{10}-KD-preferred conformational state argues that des-Arg^{10}-KD is a *bona fide* B1 receptor agonist and that the receptor does not solely rely on constitutive activity for function. The sensitivity of the B1 receptor to the mutation of this residue argues that the normal elevated activity of the receptor is not due to the misalignment of Asn^{121}.

IC-IV represents another domain that may be responsible for regulating constitutive receptor activity. Epitopes that interact with effectors involved in both signaling (*e.g.* G protein) and desensitization (*e.g.* GPCR kinase and arrestin) are generally present in this domain, and both have been proposed to influence ligand-independent GPCR activity. Thus, receptor activity should be sensitive to the presence and absence of receptor

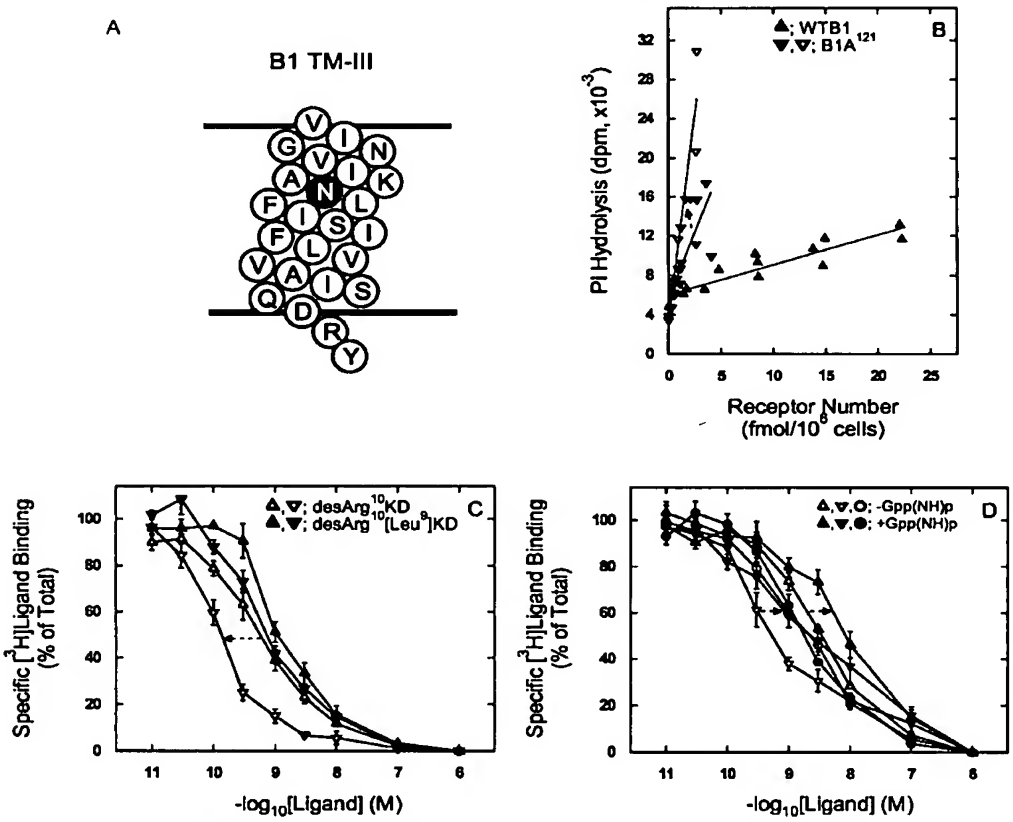

FIG. 5. **Ligand-independent and agonist-dependent PI hydrolysis and agonist binding by the human B1 receptor and B1A^{121}.** *A*, the amino acid sequence of TM-III of the human B1 receptor is shown. Indicated is Asn^{121} (*filled circle*). *B*, HEK293 cells transfected with varying amounts of the WT B1 receptors (▲) or B1A^{121} (▼, ▽) were incubated in the absence (*closed symbols*) and presence (*open symbols*) of 1 μM des-Arg^{10}-KD as indicated and then assayed for PI hydrolysis and radioligand binding using a saturating concentration of radioligand. The effect of des-Arg^{10}-KD is indicated (*dashed arrow*). The results are from 4 to 8 experiments with each point performed in duplicate. *C*, particulate preparations of cells transfected with WT B1 receptors (△, ▲) or B1A^{121} (▽, ▼) were washed three times, incubated in the absence and presence of increasing concentrations of des-Arg^{10}-KD (△, ▽) or des-Arg^{10}-[Leu^9]KD (▲, ▼) as indicated, and assayed for saturation binding using a K_d concentration of [^{3}H]des-Arg^{10}-KD and [^{3}H]des-Arg^{10}-[Leu^9]KD, respectively. The effect of the Asn^{121} → Ala mutation on des-Arg^{10}-KD binding is indicated (*dashed arrow*). The results are averages ± S.E. of three independent experiments with each point assayed in duplicate. Binding constants (K_d) calculated from these experiments are summarized in Table II. *D*, particulate preparations of cells transfected with WT B1 receptors (△, ▲, ○, ●) or B1A^{121} (▽, ▼) were washed three times, incubated in the absence and presence of increasing concentrations of des-Arg^{10}-KD (△, ▲, ▽, ▼) and des-Arg^{10}[Leu^9]KD (○, ●), with and without 100 μM Gpp(NH)p as indicated, and assayed for competition binding using K_d concentrations of [^{3}H]des-Arg^{10}-[Leu^9]KD and [^{3}H]des-Arg^{10}-KD, respectively. The effects of Gpp(NH)p on des-Arg^{10}-KD binding to WT B1 receptors and B1A^{121} are indicated (*dashed arrow*). The results are averages ± S.E. of three independent experiments with each point assayed in duplicate. Binding constants (K_i) calculated from these experiments are summarized in Table II.

TABLE II
Binding constants of B1 receptor peptide agonists and antagonists on WT B1 receptors and B1A^{121}[a]

Ligand	WT B1	B1A^{121}	WT B1		B1A^{121}	
			−Gpp(NH)p	+Gpp(NH)p	−Gpp(NH)p	+Gpp(NH)p
	K_d (nM)[b]		K_i (nM)[c]			
Des-Arg^{10}KD	1.67 ± 0.27	0.504 ± 0.085[d]	3.00 ± 0.05	6.45 ± 0.92[e]	0.547 ± 0.264[d]	1.17 ± 0.30
Des-Arg^{10}[Leu^9]KD	2.06 ± 0.59	1.25 ± 0.30	1.90 ± 0.17	1.85 ± 0.25		

[a] Particulate preparations of transiently transfected cells were washed three times prior to assay.
[b] The K_d values were determined by the Radlig program in "cold" saturation binding experiments (Fig. 5*C*) using a constant concentration of [^{3}H]des-Arg^{10}-KD and [^{3}H]des-Arg^{10}[Leu^9]KD and increasing concentrations of des-Arg^{10}-KD and des-Arg^{10}[Leu^9]KD, respectively. The values are presented as the average ± S.E. of at least three experiments.
[c] The K_i values were determined by the Radlig program in competition binding experiments (Fig. 5*D*) using a constant concentration of [^{3}H]des-Arg^{10}-KD and [^{3}H]des-Arg^{10}[Leu^9]KD and increasing concentrations of des-Arg^{10}[Leu^9]KD and des-Arg^{10}KD, respectively, in the absence and presence of 100 μM Gpp(NH)p. The values are presented as the average ± S.E. of at least three experiments.
[d] Significantly different from the corresponding value for WT B1, $p < 0.05$.
[e] Significantly different from the corresponding value in the absence of Gpp(NH)p, $p < 0.05$.

epitopes through which such effectors interact. Indeed, we recently used the B2 receptor to provide evidence for a role of IC-IV in regulation of ligand-independent receptor activity (31). This receptor exhibits minimal ligand-independent activity. However, a significant rise in activity was observed following alanine mutation of a cluster of serines and threonines in IC-IV of this receptor. This cluster is a substrate for agonist-promoted receptor phosphorylation (32) and important for B2

FIG. 6. **Amino acid sequence alignment of IC-IV of B2 and B1 receptors from different species.** Indicated is the predicted junction of the seventh transmembrane domain and IC-IV (*vertical line*), species conservation of residues in each receptor subtype (*bold*), species conservation of serines and threonines in each receptor receptor subtype (*), and the cluster of serines and threonines conserved among B2 receptors from different species that were mutated into alanines to create the receptor constructs B1(B2ICIVA$^{Ser/Thr}$) and B1(B2ICIVA$^{Ser/Thr}$)A^{121} (*Ser/Thr cluster*). The numbering of residues in the B2 and B1 receptors is according to Hess *et al.* (1) and Menke *et al.* (2).

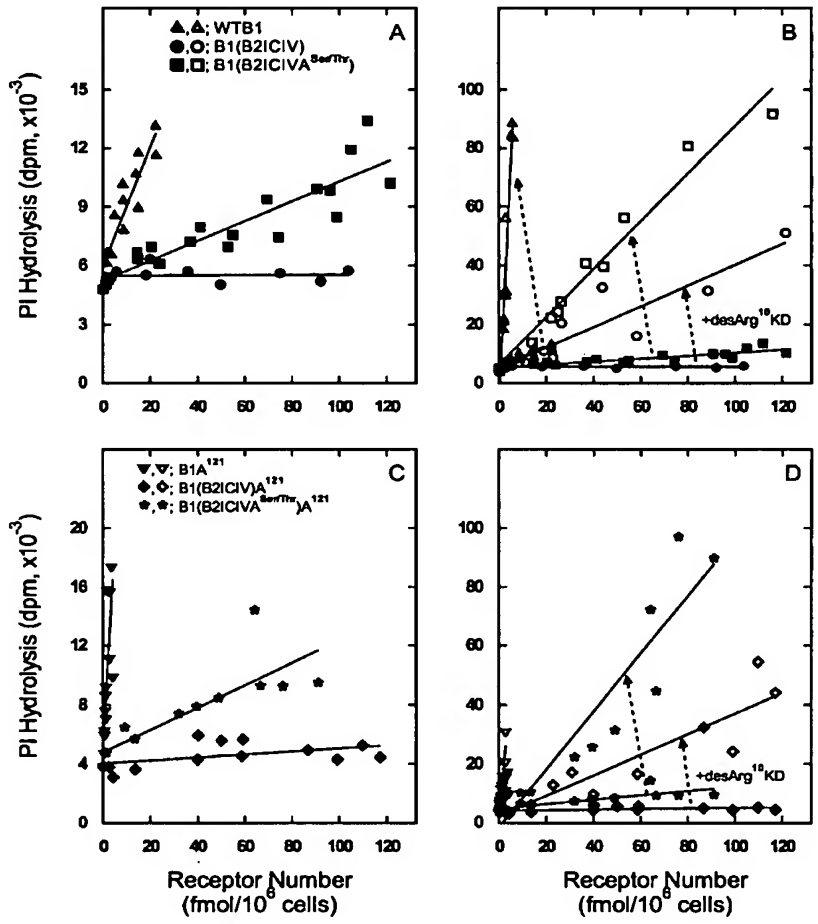

FIG. 7. **Ligand-independent PI hydrolysis by the human B1/B2 receptor IC-IV chimeras.** *A* and *B*, HEK293 cells transfected with varying amounts of the WT B1 (▲, △), B1(B2ICIV) (●, ○), or B1(B2ICIVA$^{Ser/Thr}$) (■, □) were incubated in the absence (*A* and *B*, *closed symbols*) and presence (*B*, *open symbols*) of 1 μM des-Arg^{10}-KD as indicated and then assayed for PI hydrolysis and radioligand binding using a saturating concentration of radioligand. *C* and *D*, cells transfected with varying amounts of B1A^{121} (▼, ▽), B1(B2ICIV)A^{121} (◆, ◇), or B1(B2ICIVA$^{Ser/Thr}$)A^{121} (★, ☆) were incubated in the absence (*C* and *D*, *closed symbols*) and presence (*D*, *open symbols*) of 1 μM des-Arg^{10}-KD as indicated and then assayed for PI hydrolysis and radioligand binding using a saturating concentration of radioligand. *B* and *D*, the effects of des-Arg^{10}-KD are shown (*dashed arrows*). Note the difference in the *y* axis scales. The results are from 3 to 8 independent experiments with each point performed in duplicate.

receptor desensitization (31). Thus, the Ser/Thr cluster exerts a suppressive effect on the ligand-independent activity of this receptor. The presence of other suppressive epitopes in IC-IV of this receptor was not readily obvious since the maximal level of ligand-independent B2 receptor activity is unknown.

The human B1 receptor contains several serines and threonines in IC-IV, but none of them are conserved in B1 receptors from different species. In fact, the B1 receptor is apparently not phosphorylated (35), and it is subject to very limited desensitization (16) and internalization (38). The dramatic inhibition by B2 receptor IC-IV on the constitutive activity of the WT B1 receptor and B1A^{121} reiterates the suppressive effect of this domain. Furthermore, this effect provides us with a crude sense of the upper and lower limits of activity of this receptor. Subsequent mutation of the Ser/Thr cluster in this chimera partially restored the constitutive activity. This gain-of-function mutation corroborates that serines and threonines are important epitopes for regulating constitutive activity. However, equally important is that these sequential loss- and gain-of-function mutations reveal the existence of an additional, currently unknown, epitope(s) in B2 receptor IC-IV that regulate ligand-independent receptor activity. Furthermore, this

regulation occurs independently of the state of receptor activation since it was observed with the constitutive activity of both the WT B1 receptor and $B1A^{121}$. Taken together, we conclude that the high constitutive activity of the B1 receptor is at least in part a consequence of the lack of epitopes in IC-IV that regulate such activity.

Although our understanding of the precise physiological and pathophysiological role of the B1 receptor is still relatively sparse, past studies have indicated that this receptor is generally silent or absent in healthy tissues but is induced in response to pathological insults during which it mediates cardiovascular and nociceptive responses to the insult (7). The recent study of mice lacking the B1 receptor gene has confirmed past conclusions as well as shed new light on the significance of this receptor (14). These mice are healthy, fertile, and normotensive. On the other hand, endotoxin-induced hypotension and accumulation of polymorphonuclear leukocytes in inflamed tissue is dramatically attenuated. B1 receptors apparently are not involved in the noxious heat sensitivity of isolated nociceptors but instead facilitate a more complex nociceptive reflex. Further evidence for the pathophysiological role of the B1 receptor has come from the identification of two allelic polymorphisms of this receptor (39, 40). One of these polymorphisms involves a G → C substitution in a positive control region of the promoter, and the C allele yields an increase in the activity of the B1 receptor promoter. Interestingly, the C allele is significantly less prevalent in individuals with end-stage renal failure and inflammatory bowel disease.

The interest in using the B1 receptor as a target for novel anti-inflammatory agents has grown with the understanding of the function of this receptor in inflammation and nociception (41). A number of B1 antagonists are available, even though all of them are peptides and structurally highly related. Several of these antagonists exhibit remarkably high affinities and selectivities for the B1 receptor. The identification of constitutive B1 receptor activity prompted the necessity to investigate inverse agonism in the interaction of these antagonists with this receptor. In receptor models, this pharmacological parameter is defined as the ability of a ligand to bind preferentially to and stabilize the inactive state of the receptor and, consequently, inhibit not only agonist-dependent activity but also ligand-independent activity of the receptor. This action is in contrast to neutral antagonists, which are nonselective for various receptor states and, consequently, only inhibit agonist-dependent activity. None of the antagonists significantly inhibited the constitutive B1 receptor activity even though des-Arg^9-[Leu^8]BK, des-Arg^{10}-[Leu^9]KD, and NPC18565 seem to elicit a small effect. Thus, available B1 receptor antagonists are either neutral or very weakly efficacious inverse agonists. It is appropriate to briefly discuss the effect of NPC18565 and des-Arg^{10}-HOE140 since these antagonists were derived from the high affinity and selective B2 receptor antagonists NPC17731 and HOE140, respectively, by simply truncating the C-terminal arginine. The parent B2 antagonists act as highly efficacious inverse agonists on the B2 receptor (23, 31, 42). In addition, Gpp(NH)p increases the potency of these ligands to compete for [^{3}H]BK binding indicating that they stabilize the G protein-uncoupled state of the B2 receptor. The lack of significant inverse agonism of des-Arg^{10}-[Leu^9]KD was reinforced by the fact that the binding of this antagonist was not perturbed by Gpp(NH)p. Considering the constitutive activity of the B1 receptor described in this report, it is clear that inverse agonism has to be considered seriously in the search for effective anti-inflammatory agents acting through the B1 receptor. Furthermore, inverse agonists will be key to the identification of constitutive activity of native B1 receptors *in vitro* and *in vivo*.

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